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Vanillin-derived antiproliferative compounds influence Plk1 activity

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ABSTRACT

We synthesized a series of vanillin-derived compounds and analyzed them in HeLa cells for their effects on the proliferation of cancer cells. The molecules are derivatives of the lead compound SBE13, a potent inhibitor of the inactive conformation of human polo-like kinase 1 (Plk1). Some of the new designs were able to inhibit cancer cell proliferation to a similar extent as the lead structure. Two of the compounds ((({4-[(6-chloropyridin-3-yl)methoxy]-3-methoxyphenyl}methyl)(pyridin-4-ylmethyl)amine) and (({4-[(4-chlorophenyl)methoxy]-3-methoxyphenyl}methyl)(pyridin-4-ylmethyl)amine)) were much stronger in their capacity to reduce HeLa cell proliferation and turned out to potently induce apoptosis and reduce Plk1 kinase activity in vitro.

Natural products and their derivatives are one of the major sources for novel antiproliferative compounds, exhibiting various modes of cytostatic action.[1] Vanilloids have been described as a valuable compound class exhibiting potential anticancer properties. In a previous study, we identified a vanillin derivative **13** (SBE13) as a potent Human polo-like kinase 1 (Plk1) inhibitor.[2] It displays selectivity towards the family members Plk2 and Plk3 known as tumor suppressor genes. Plk1,[3] a key regulator of mitosis, is over-expressed in all today analyzed human tumors.[4–6] Its expression level is a negative prognostic and predictive factor for cancer patients and serves as a measure of the aggressiveness of a tumor.[6,7] Thus, targeting Plk1 by small molecules might be a promising approach to cancer therapy, and Plk1 inhibitors represent tools for cancer research and for the mechanistic investigation of checkpoint control.[8–10] Several chemical entities targeting Plk1 already reached clinical trials (Scheme 1). The dihydropteridinone-derived inhibitors BI2536[11,12] and BI6727 (volasertib)[13] are the most advanced clinical candidates. BI2536 and BI6727 are highly potent ATP-competitive Plk1 inhibitors (BI2536 IC_{50} = 0.83 nM, BI6727 IC_{50} = 0.87 nM), which do not discriminate between the different Plk family members. BI6727 is currently being investigated in phase II clinical trials as monotherapy or combined with established chemotherapeutic agents.[14] Chemically related agents TAK-960[15] and NMS-P937[16,17] are currently under phase I investigation. The thiophene benzimidazole GSK461364[18] has been investigated in a phase I study in patients with advanced solid tumors.[19] Two ATP-noncompetitive inhibitors, ON01910[20] and HMN-214,[21] have shown efficacy in phase I trials and are currently under advanced clinical development.

In this study, we analyzed the structure activity relationship (SAR) of antiproliferative vanillin derivatives derived from compound **13** (**SBE13**). We first screened analogs of compound **13**, which were commercially available from the Specs compound library (Specs Int., The Netherlands) and featured an *ortho*-dimethoxyphenylethylamine moiety (Table 1). We subdivided these structures in three groups.

The first group (**1**–**5**) consists of structures with nonaromatic amine substituents. The cyclohexane-derivative **3** was insoluble in concentrations required for cellular assays, the introduction of ethyl methyl ether (**2**, EC_{50} = 97 μM), morpholine (**4**, EC_{50} > 100 μM) and tetrahydrofuran (**5**, EC_{50} = 66 μM) resulted in loss of activity. However, the substitution with the propyl dimethylamine (**1**, EC_{50} = 5 μM) restored inhibitory potency.

The second group comprises derivatives of compound **13** with phenylethyl and benzyl substituents (**6**–**14**, **19**). The *para*-methoxybenzene (**8**) and the benzoic acid (**12**) were insoluble. Removal of both methoxy groups yielded a less potent compound **6** (EC_{50} = 61 μM) suggesting the importance of hydrogen-bond acceptor functionality in the aromatic ring. Potency could not be

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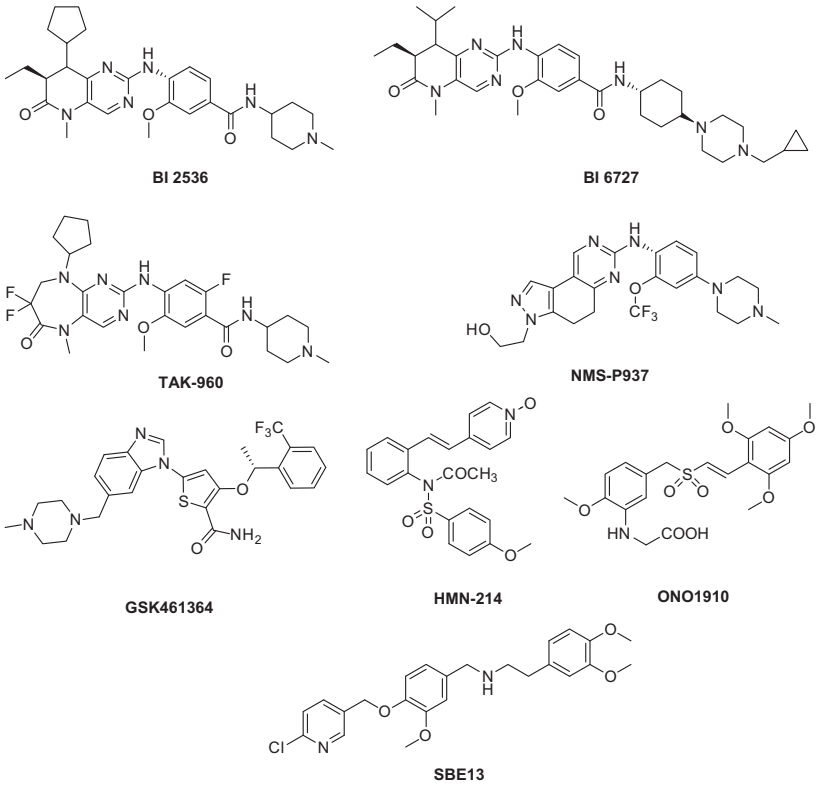
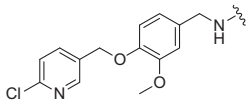
0960-894X/



Scheme 1.

Table 1
Variation of the amine residue of the SBE13 lead structure (**13**)



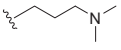
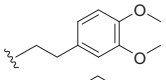
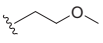
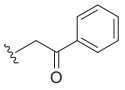
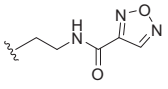
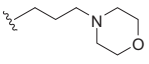
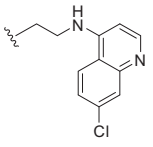
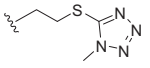
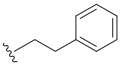
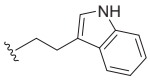
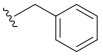
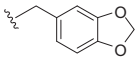
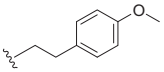
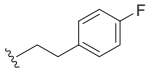
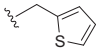
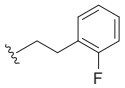
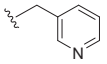
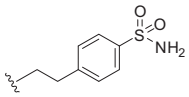
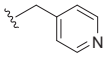
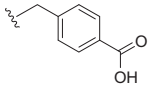
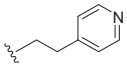
Compound no.	R	EC_{50} (μM)	Compound no.	R	EC_{50} (μM)
1		5	**13**		18
2		97	**14**		60
3		Insoluble	**15**		24
4		>100	**16**		2.7
5		66	**17**		86
6		61	**18**		8
7		90	**19**		71
8		Insoluble	**20**		69

Table 1 (*continued*)

Compound no.	R	EC_{50} (μM)	Compound no.	R	EC_{50} (μM)
9		16	**21**		96
10		18	**22**		5
11		12	**23**		0.4
12		Insoluble	**24**		55

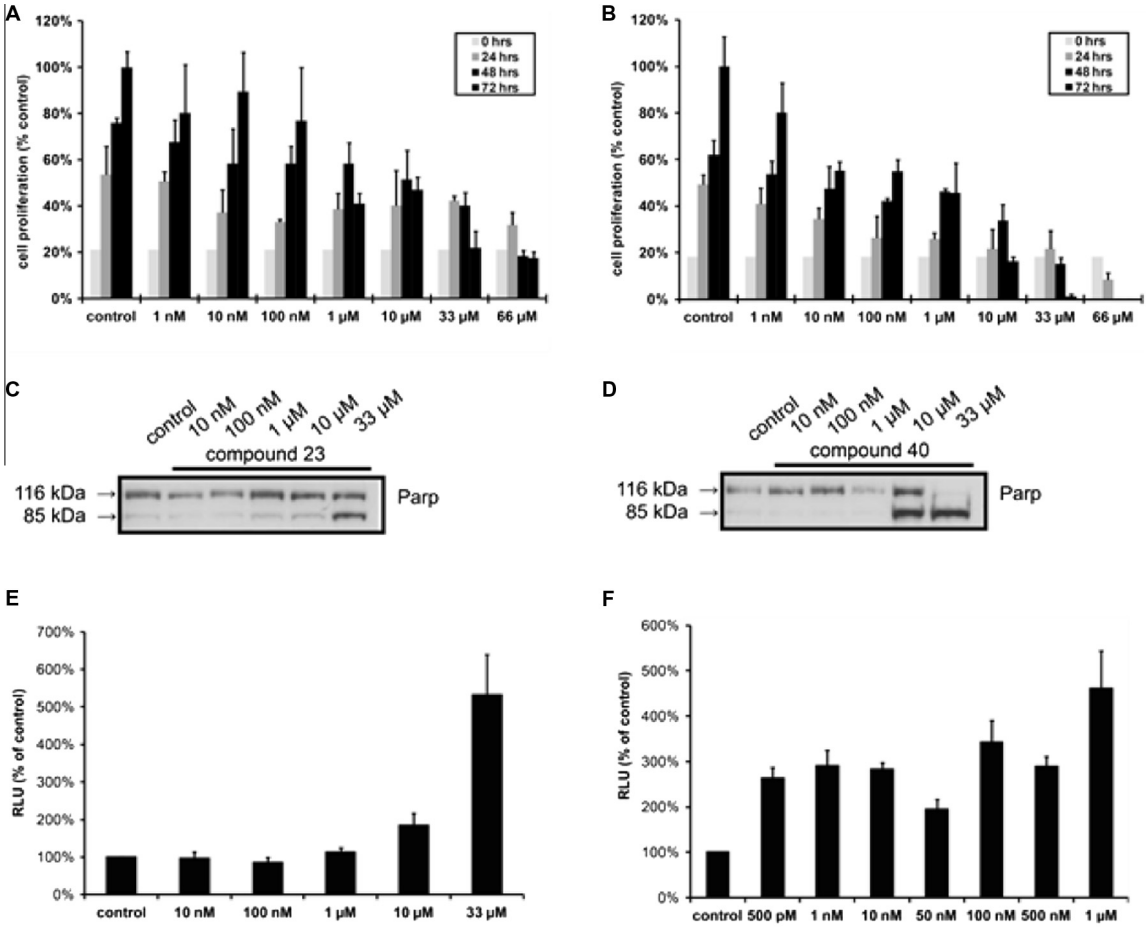
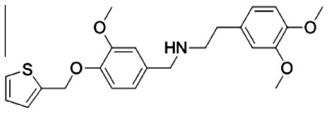
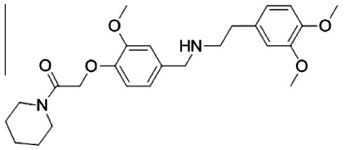
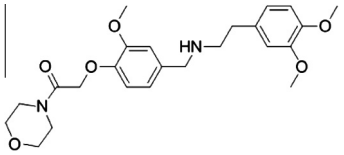
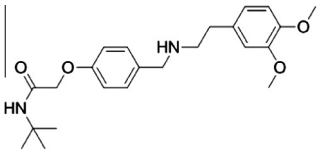
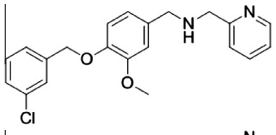
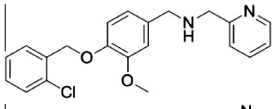
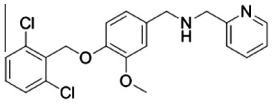
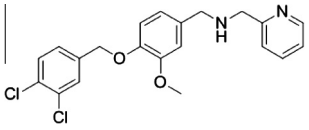


Figure 1. Cell proliferation and apoptosis. Effect of compound **23** (A) and **40** (B) on cell proliferation and apoptosis in HeLa cells. Cell proliferation of HeLa cells was analyzed 24–72 h after treatment. Control cells were incubated with culture medium alone. Percentage of surviving cells is given as percentage of the number of control cells 72 h after incubation with the inhibitors. All experiments: $n = 3$. Western blot analysis of Parp cleavage after treatment of HeLa cells with compound **23** (C) and compound **40** (D). To determine the full-length Parp protein and the cleavage product in apoptotic cells, Western blot analyses targeting Parp were performed in HeLa cells 48 h after incubation. Caspase 3/7 activation was monitored 48 h after treatment with compound **23** (E) and compound **40** (F), respectively. Luminescence is given as relative RLU levels ($n = 3$, mean ± SD). Control cells were incubated with normal culture medium.

restored by the introduction of the carbonyl group as present in compound **14** (EC_{50} = 60 μM). This result is in line with the potency yielded by compounds **9** (EC_{50} = 16 μM) and **11** (EC_{50} = 12 μM), with this functionality restored by the introduction of fluorine or a sulphonamide moiety, respectively. Surprisingly, activity was also restored by the introduction of fluorine in *ortho*-position (**10**, EC_{50} = 18 μM). Shortening the ethyl linker by one carbon (compound **7**, EC_{50} = 90 μM) almost fully removed anti-proliferative activity.

We examined the tolerability of the third group consisting of diverse heterocyclic amino substituents that were available from the commercial supplier. A thiophene analog of **7**, compound **21**, (EC_{50} = 96 μM) was almost inactive, as well as the *N*-methyl tetrazole- (**17**, EC_{50} = 86 μM), 1,3-benzodioxole- (**19**, EC_{50} = 71 μM),

Table 2
Variation of the ether residue of the lead structure **13**

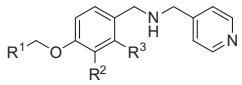
Compound no.	Structure	EC_{50} (μM)
25		18
26		87
27		85
28		>100
29		18
30		22
31		6.1
32		13

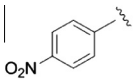
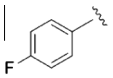
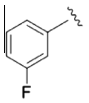
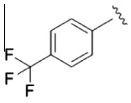
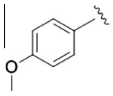
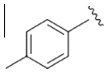
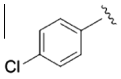
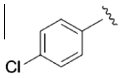
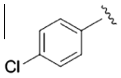
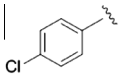
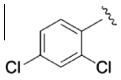
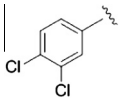
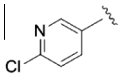
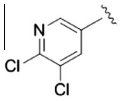
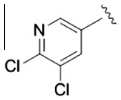
1,2,3-triazole- (**20**, EC_{50} = 69 μM) bearing structures. The 1,2,5-oxadiazole amide moiety (**15**, EC_{50} = 24 μM) led to a slightly decreased inhibition compared to **13**. Activity was restored by the introduction of the tryptamine (**18**, EC_{50} = 8 μM) as well as the more complex 4-amino-7-chloroquinoline (**16**, EC_{50} = 2.7 μM). Replacement of benzene (**7**) by 3-pyridine (**22**, EC_{50} = 5 μM) completely restored the potency, and the 4-pyridine substituent led to 45-fold improvement (**23**, EC_{50} = 0.4 μM, Fig. 1A), thereby yielding a novel lead structure for further optimization. The extension of the linker length by one methylene resulted in a loss of potency (**24**, EC_{50} = 55 μM).

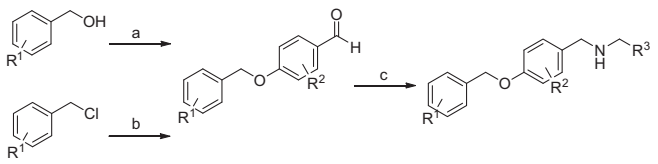
We further examined the impact of the ether substituent by screening a set of commercially available compounds with either 3,4-dimethoxyphenylethylamine or 2-aminomethyl-pyridine in the eastern part of compound **13** (Table 2). Thiophene successfully replaced the 2-chloropyridine, yielding compound **25** (EC_{50} = 18 μM). The introduction of more polar amide substituents **26**–**28** led to the complete loss of potency. However, the different chloro substitution patterns (**29**–**32**) were well tolerated suggesting that optimization of the hydrophobic substitution pattern in the western part might yield highly potent antiproliferative compounds.

Prescreening led to the identification of structure **23** (EC_{50} = 0.4 μM) as a promising inhibitor. Preliminary SAR of this lead structure was explored. Our next step was the synthesis of new derivatives of compound **23** to evaluate the SAR of the central aromatic ring and the western substituent (Table 3). The synthesis was carried out according to Scheme 2. We exchanged the 2-chloropyridine by *para*-chlorobenzene (**40**, EC_{50} = 0.89 μM), which led to slightly worse inhibition. Interestingly, the hydrochloride salt of compound **40**, which was obtained from the commercial supplier Specs yielded a slightly altered EC_{50} = 0.45 μM (Fig. 1B), which might be associated to different solubility characteristics of oxalates and hydrochlorides. Then, we analyzed different substituents on the benzyl

Table 3
Optimization of compound **23**



Compound no.	R^1	R^2	R^3	EC_{50} (μM)
33			–H	0.44
34			–H	6.4
35			–H	4.8
36			–H	5.1
37			–H	2.4
38			–H	5.0
39			–H	5.0
40			–H	0.89
41		–H		0.44
42		–H	–H	4.0
43			–H	2.5
44			–H	3.9
45			–H	4.0
46		–H		3.3
47			–H	0.22
48		–H		0.40



Scheme 2. Synthesis of vanillin derivatives. (a) vanillin derivative (1 equiv), alcohol (1.2 equiv), TPP (1.2 equiv), DEAD (1.2 equiv), THF, 0 °C, overnight. (b) Vanillin derivative (1 equiv), halogenide (1.2 equiv), Cs_2CO_3 (1 equiv), DMF, 70 °C, 2 h. (c) Aldehyde (1 equiv), amine (1 equiv), DCE, room temperature, 3 h, then $NaBH(Ac)_3$ (1.2 equiv), room temperature, overnight.

moiety. We investigated the impact of different substituents in *para*-position. The *p*-fluorobenzyl (**34**, EC_{50} = 6.4 μM), *p*-trifluorobenzyl (**37**, EC_{50} = 2.4 μM), *p*-methylbenzyl (**39**, EC_{50} = 5 μM) and *p*-methoxybenzyl (**38**, EC_{50} = 5 μM) substituents were less active. Only the *p*-nitrobenzyl (**33**, EC_{50} = 0.44 μM) moiety demonstrated a comparable inhibitory effect. The next step was a fluorine scan: the *m*-flourobenzyl (**35**, EC_{50} = 4.8 μM) and *o*-fluorobenzyl (**36**, EC_{50} = 5.1 μM) exhibited potency comparable to the fluorine substituent in *para*-position. Next we extended the *para*-chlorobenzyl substituent by chlorine in *ortho*- (**44**, EC_{50} = 3.9 μM) and in *meta*-position (**45**, EC_{50} = 4 μM). The addition of a second chlorine afforded less potent compounds. Interestingly, the introduction of nitrogen (**47**, EC_{50} = 0.22 μM) improved potency.

After optimization of the western substituent we analyzed the impact of the methoxy group in the central aromatic ring. The removal of the methoxy group (**42**, EC_{50} = 4 μM) as well as the exchange of the methoxy with the ethoxy (**43**, EC_{50} = 2.5 μM) moiety resulted in weaker inhibitors. However, the transition of the methoxy substituent from *ortho*- to *meta*-position (**41**, EC_{50} = 0.4 μM) restored the inhibitory potency. The same transition applied to the methoxy group in the pyridine-substituted compounds (**23** and **47**) led to opposite results: We observed that transferring the methoxy group from *ortho*- to *meta*-position led to a weaker inhibition (**48**, EC_{50} = 0.4 μM; **46**, EC_{50} = 3.3 μM).

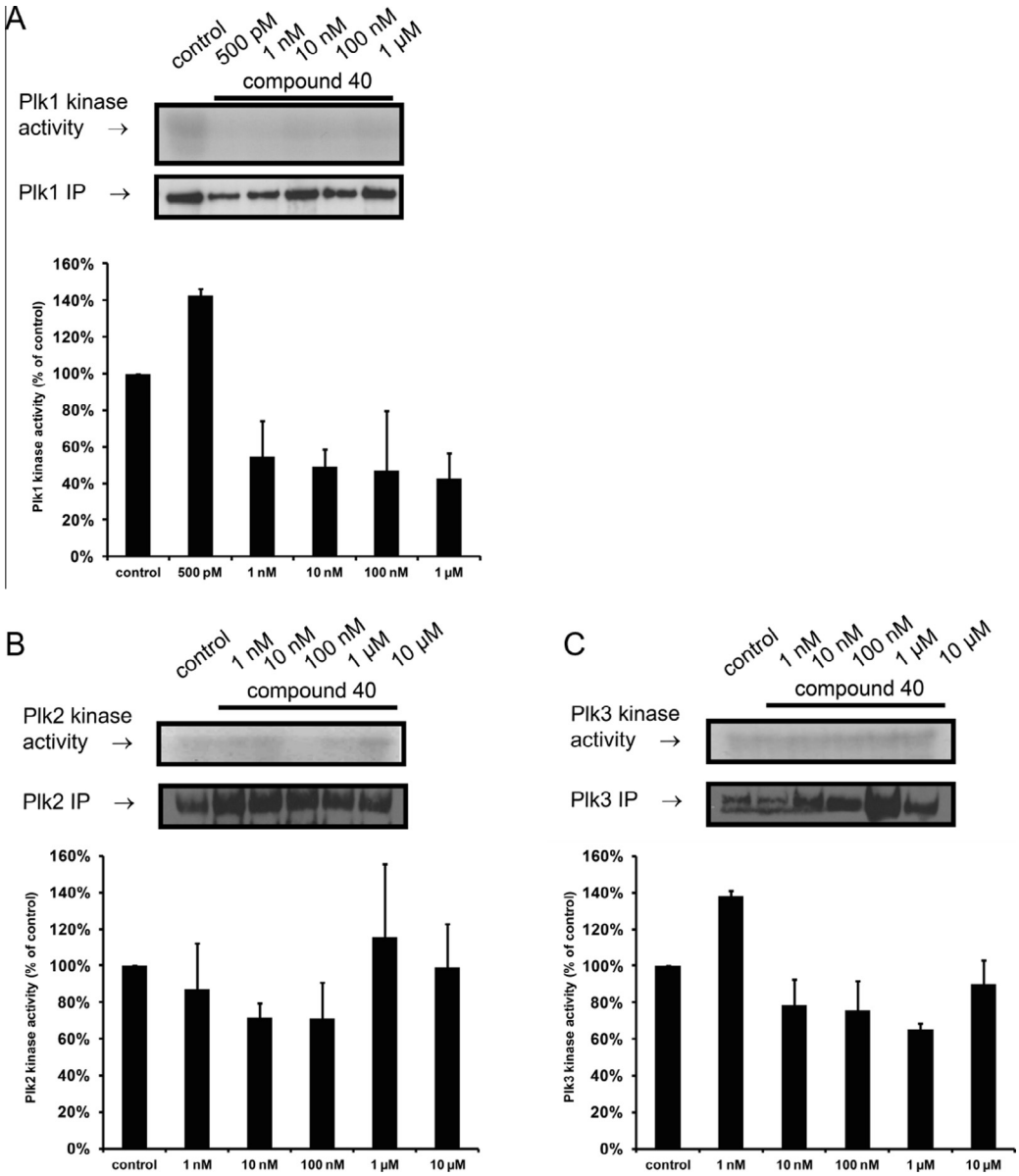


Figure 2. Determination of Plk1, Plk2 and Plk3 kinase activity. Kinase assays using immunoprecipitated Plk1 (A), Plk2 (B) or Plk3 (C), respectively, from HeLa cells after incubation with compound **40**. The autoradiograms show representative assays measuring kinase activity after immunoprecipitation of Plks from HeLa cells after incubation with the compound, followed by subsequent kinase assay using casein as a substrate.

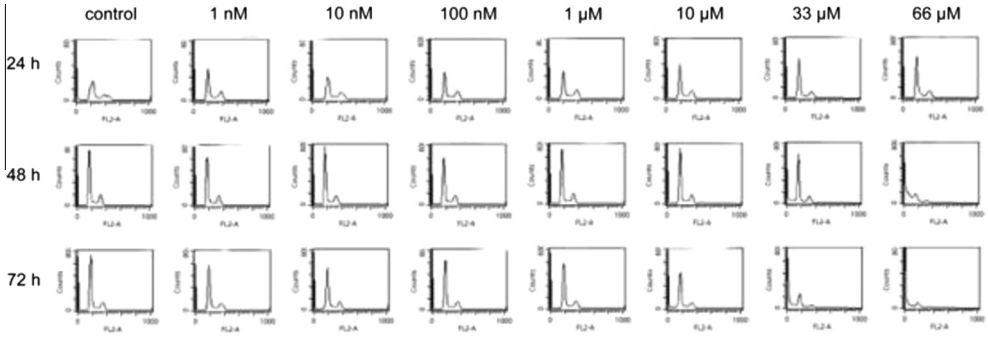


Figure 3. Cell-cycle progression. Effect of compound **40** on the cell cycle distribution of HeLa cells. FACScan analyses were done after 24, 48, and 72 h incubation with the compound. The graphs show the absolute number of cells in the respective cell cycle phases. The left peak represents cells in G0/G1 phase, and the right peak represents cells in G2/M phase. The sub-G0/G1 peak is detectable at higher concentrations and later time points.

Taken together, from cell proliferation analyses in combination with the additional visual inspection of cell morphology, two derivatives of compound **13** (compounds **23** and **40**,) turned out to be potent Plk1 inhibitors. Other derivatives (**33**, **41**, **47** and **48**), which yielded submicromolar antiproliferative potency, exhibited atypical cell morphology for selective Plk1 inhibition. Therefore, further analyses were performed with compounds **23** and **40**.

To investigate whether the reduced cell proliferation after treatment with the two selected derivatives of compound **13** (compound **23** and **40**, respectively) might be due to apoptosis we first did Western blot analyses against Parp in HeLa cells 48 h after treatment. We were able to detect an increasing amount of cleavage products of 85 kDa, accompanied by a decrease of the full length protein of 116 kDa, after treatment with compounds **23** and **40**, where complete degraded full length protein was observed for compound **40** with 33 μM (Fig. 1C and D). To confirm the induction of apoptosis we did Caspase 3/7 assays (Fig. 1E and F). Here we observed a concentration-dependent induction of apoptosis after treatment with both compounds, which was more prominent with compound **40** than with compound **23**. After treatment of HeLa cells with compound **23** the induction of apoptosis was only significant for the two highest concentrations of 10 μM and 33 μM ($p = 0.04$ and $p = 0.02$, respectively). Compound **40** induced apoptosis more strongly than compound **23**, which was significant already at the lowest concentration of 500 pM ($p = 0.007$).

To analyze whether compound **40**, which turned out to be potent in first cell culture studies using HeLa cells, inhibits Plk1 kinase activity, we performed kinase assays using immunoprecipitated Plk1 kinase from synchronized and treated HeLa cells. Compound **40** inhibited Plk1 kinase activity with an IC_{50} value of 3 nM (Fig. 2A). Reduction of kinase activity was statistically significant at all tested concentrations (500 pM–1 μM, p <0.01). Next we checked whether compound **40** also discriminates between the Plk family members. For that reason Plk2 and Plk3 kinase activity was analyzed (Fig. 2B and C). Compound **40** also showed selectivity towards Plk2 and Plk3 with IC_{50} values >10 μM for both kinases.

To analyze the cell cycle distribution and to confirm the apoptosis induction observed in Caspase 3/7 assays we did FACS analyses using compound **40** at time points 24–72 h after treatment (Fig. 3). As observed in Caspase 3/7 assays we observed a concentration- and time-dependent induction of apoptosis.

In this study, we investigated SARs of vanillin derivatives exhibiting antiproliferative effects. We started from our lead compound **13**[2] and subsequently optimized its three aromatic systems as well as the length of the amine linker. Several compounds yielded twofold greater potency than compound **13** in cell-based assays. A blueprint of an effective vanillin derivative can be deduced from the SAR. It consists of a chlorinated pyridinyl or phenyl moiety coupled via benzylic ether to the vanillin core. The methoxy substituent at the central aromatic ring is apparently required for the activity. However the effect of the methoxy substituent depends on the ether substituent. The SAR of the amine substituent is restrictive considering both, linker length and type of heterocycle. A chain length of one carbon atom and 4-pyridine are suitable to yield active compounds.

Our optimization strategy yielded several compounds (**23**, **33**, **40**, **41**, **47**, **48**) with submicromolar activity in cellular assays. Visual inspection of the phenotype of the treated cells implied that the antiproliferative effect of compounds **23** and **40** is mainly based on Plk1 inhibition. Consequently we proceeded with the characterization of **23** and **40** in order to test the proposed mode of action via kinase assay using immunoprecipitated Plk1, Plk2 and Plk3, cell cycle analysis and PARP cleavage analysis which revealed a strong selective inhibition of Plk1 activity and induction of apoptosis. These observations suggest compound **23** and **40** as new potent Plk1 inhibitors with nanomolar inhibitory effect on cell proliferation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.09.015.

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